Molecular Dynamics Simulations of Leu-Enkephalin in Water and DMSO

David van der Spoel and Herman J. C. Berendsen
Bioson Research Institute and Laboratory of Biophysical Chemistry, University of Groningen, Groningen, The Netherlands

ABSTRACT The structure of Leu-enkephalin (L-Enk) and Met-enkephalin (M-Enk) have frequently been studied, in particular by nuclear magnetic resonance spectroscopy. After more than 20 years of research, it was concluded that enkephalins have no preferred structure in aqueous solution, but that they may have in other solvents. We have performed molecular dynamics simulations of zwitterionic L-Enk in water, and zwitterionic as well as neutral L-Enk dimethyl sulfoxide (DMSO). In water the peptide is very flexible, although there seems to be a preference for compact conformations. In DMSO, the peptide forms a clear salt bridge in the zwitterionic form, but has no preferred conformation in the neutral form. This difference in conformation may provide an explanation for measurements in DMSO in which multiple conformations were found to exist. In this paper we introduce a new formulation for a dihedral angle autocorrelation function, and apply it to study side-chain dynamics in L-Enk. We find that the side-chain dynamics of the large Tyr and Phe residues cannot be adequately sampled in 2.0-ns simulations, while this does seem to be possible for the smaller Leu side chain.

INTRODUCTION

The study of the conformation of enkephalin in solution is an interesting case for those interested in the history of biophysical chemistry; it contains many contradictions and erroneous measurements, and, as of the present, the picture is still unclear. It also nicely demonstrates the advance of experimental techniques aimed at resolving the structure of such peptides over time.

In 1975 it was first reported that small molecules in the brain named enkephalins, with opioid activity, which had been known for some time, were in fact pentapeptides (Hughes et al., 1975). It was found by using mass spectrometry that two different peptides were present with the sequence Tyr-Gly-Gly-Phe-[Leu/Met] in a ratio of 3 or 4 Met to 1 Leu (Hughes et al., 1975). Since this discovery, scientists have tried to determine the conformation of these peptides in different solvents such as water and dimethyl sulfoxide (DMSO), using spectroscopic techniques, predominantly nuclear magnetic resonance (NMR) spectroscopy, but also circular dichroism (CD) and Fourier transform-infrared (FTIR) spectroscopy (Khaled et al., 1977; Garbay-Jaureguiberry et al., 1977; Stimson et al., 1979; Higashijima et al., 1979; Gupta et al., 1986; Motta et al., 1987a,b; 1988; Gerothanassis et al., 1987; Surewicz and Mantsch, 1988; Glasser and Scheraga, 1988; Sakarellos et al., 1989; Vesterman et al., 1989; Picone et al., 1990; Moret et al., 1990; Gerothanassis et al., 1992; Graham et al., 1992; Doi et al., 1994). In 1977 Khaled et al. found that some of the physical properties of Met-enkephalin (M-Enk) and Leu-enkephalin (L-Enk), such as proton and carbon chemical shifts and CD, were concentration-dependent in DMSO (Khaled et al., 1977). Based on this result, the authors proposed a β-sheet dimer structure with four hydrogen bonds and two salt bridges. This proposal was rejected in 1979 by Stimson et al., who were unable to reproduce the concentration-dependence of the chemical shifts (Stimson et al., 1979); the effect reported by Khaled et al. was blamed on impurities in the sample. Instead, Stimson et al. proposed a β-bend type structure for L-Enk in DMSO, with a hydrogen bond from Leu^5-NH to Gly^2-CO. In that same year Higashijima et al. concluded that M-Enk is in an equilibrium between folded and unfolded conformations in DMSO, while they could not find evidence for a folded conformation in water (Higashijima et al., 1979). 1D NOE measurements of M-Enk in D_2O were described in 1986 by Gupta et al., from which the authors concluded that the aromatic rings of Tyr and Phe are close in space (Gupta et al., 1986). However, one year later this was shown to be an artifact by Motta et al. (Motta et al., 1987b). Vesterman et al. (1989) used ROESY experiments and Monte Carlo calculations to study L-Enk in DMSO. They concluded that two separate folded conformations exist simultaneously in this solvent (Vesterman et al., 1989).

Using ^{17}O-NMR, Sakarellos et al. determined that neither the Gly^2 nor the Gly^3 carbonyl oxygen can be hydrogen bonded in aqueous solution or in a mixed acetonitrile/DMSO (4:1) solution (Sakarellos et al., 1989). In 1990 Moret et al. reported the same finding in acetone solution (Moret et al., 1990). From these observations it can be concluded that there cannot be a 2←5 β-turn in L-Enk in these solvents. Sakarellos et al. also determined that L-Enk is uncharged in their mixed CH_3CN/DMSO solution (Sakarellos et al., 1989), while a mixture of zwitterionic (40%) and neutral peptides (60%) were found in a later study (1992) which again utilized ^{17}O-NMR as well as FTIR spectroscopy (Gerothanassis et al., 1992); it may be clear that the ionic state of a small peptide in organic solvents can have a profound impact on its conformation. It

Received for publication 11 September 1996 and in final form 12 February 1997.

Address reprint requests to Dr. H. J. C. Berendsen, Dept. of Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands. Tel.: 31-50-3634323; Fax: 31-50-3634800; E-mail: berends@chem.rug.nl.

0006-3495/97/05/2032/10 $2.00

should be noted that in 1980 this same finding had already been reported by Han et al. on the basis of Raman, IR, and NMR spectroscopy (Han et al., 1980).

Interactions between the aromatic groups of L-Enk and M-Enk appear to be important at lipid interfaces. A hydrogen-bonded turn was found in the presence of ditetradecylphosphatidylcholine bilayers (Surewicz and Mantsch, 1988) as well as in the presence of SDS micelles (Graham et al., 1992). Structure refinement based on the latter experiments suggested that this conformation may be stabilized by an interaction between the aromatic groups (Graham et al., 1992).

A large number of theoretical studies on L-Enk and M-Enk have been reported (Isogai et al., 1977; Paine and Scheraga, 1985, 1986, 1987; Li and Scheraga, 1987; Nayeem et al., 1991; Smith and Pettitt, 1991a, b; Karoslaw and Scheraga, 1992; Perez et al., 1992; Meirovitch et al., 1994; Zhorov and Ananthanarayanan, 1994). Most of these were methodological papers describing optimization algorithms. Li and Scheraga performed Monte Carlo with energy minimization (MCM) on M-Enk and found a single energy minimum in vacuo (Li and Scheraga, 1987). The MCM procedure was also tested on M-Enk in water; in this case the minimized conformations of five MCM runs were all different. Perez et al. describe molecular dynamics (MD) simulations of zwitterionic M-Enk in vacuo, using a simulated annealing strategy aimed at finding low-energy conformations (Perez et al., 1992). Although the history of enkephalin research makes it clear that the environment of a peptide is of crucial importance for its conformation, to our knowledge no detailed theoretical study aimed at environmental effects has been reported to date. Such theoretical studies have been reported for other peptides in the presence of TFE (de Loof et al., 1992; van Buuren and Berendsen, 1993; Brooks III and Nilsson, 1993; Bodkin and Goodfellow, 1996) or in DMSO (Mierke and Kessler, 1991, 1993).

In the present work, we have set out to study the properties of L-Enk in water as well as DMSO using relatively long MD simulations in explicit solvent. Since it was determined that in DMSO both zwitterionic and neutral peptides exist (Gerothanassis et al., 1992), we simulated both forms of the peptide. It was recently found that the water model used in simulations of aromatic peptides may influence the conformational preferences rather drastically (van der Spoel et al., 1996b), we decided to use the simple point charge (SPC) water model (Berendsen et al., 1981). We will not pursue the phantom of the solution structure of L-Enk, but rather try to assess the range of accessible conformations qualitatively.

Furthermore, we present a detailed study of the side-chain dynamics in L-Enk. We introduce a dihedral angle analysis method, which renders a correlation time that is roughly the residence time in a single dihedral angle potential minimum. The analysis method also provides two independent methods to compute a property that we named a dihedral order parameter, S^2_D, which is the autocorrelation at infinite time. Convergence of the two computation methods to the same S^2_D value provides strong evidence that the dynamics of this particular angle have equilibrated within the simulation.

THEORY

To study torsion angle dynamics we define a dihedral autocorrelation function as:

$$C(t) = \langle \cos[\theta(\tau) - \theta(\tau + t)] \rangle_\tau \tag{1}$$

Note that this is not a product of two functions as is generally used for correlation functions, but it may be rewritten as the sum of two products:

$$C(t) = \langle \cos[\theta(\tau)]\cos[\theta(\tau + t)] + \sin[\theta(\tau)]\sin[\theta(\tau + t)] \rangle_\tau \tag{2}$$

Using Eq. 1 rather than, for instance, the cosine part of Eq. 2, has the clear advantage that the correlation at time zero can be directly evaluated:

$$C(0) = 1 \tag{3}$$

When we know the dihedral distribution $p(\theta)$ we can also calculate the correlation at infinite time:

$$C(\infty) = \int_0^{2\pi}\int_0^{2\pi} \cos(\theta_1 - \theta_2)p(\theta_1)p(\theta_2)\, d\theta_1\, d\theta 2 \tag{4}$$

which can be rewritten as:

$$C(\infty) = \left[\int_0^{2\pi} \cos(\theta)p(\theta)\, d\theta\right]^2 + \left[\int_0^{2\pi} \sin(\theta)p(\theta)\, d\theta\right]^2 \tag{5}$$

Note that $0 \le C(\infty) \le 1$, since it is a sum of two squares. Analogous to the well-known model-free approach to NMR relaxation measurements of Lipari and Szabo (1982) we equate $C(\infty)$ to a dihedral order parameter S^2_D:

$$S^2_D = C(\infty) \tag{6}$$

In Table 1 we have printed the dihedral order parameters S^2_D

TABLE 1 Some normalized dihedral probability functions $p(\theta)$, and the corresponding dihedral order parameters S^2_D evaluated from Eq. 5

$p(\theta)$	Domain#	S^2_D	
$\delta(\theta)$*		1	1.0
$(1 + \cos 3\theta) \cdot 3/(2\pi)$	$0 \le \theta < 2\pi/3$	$3^7/(2^8\pi^2)$	0.865584
$3/(2\pi)$		$3^3/(2^2\pi^2)$	0.683918
$\delta[\theta \bmod (2\pi/3)]/2$		1/4	0.25
$(1 + \cos 3\theta) \cdot 3/(4\pi)$	$0 \le \theta < 4\pi/3$	$3^7/(2^{10}\pi^2)$	0.216396
$3/(4\pi)$		$3^3/(2^4\pi^2)$	0.17097
$\delta[\theta \bmod (2\pi/3)]/3$		0	0.0
$(1 + \cos 3\theta)/(2\pi)$	$0 \le \theta < 2\pi$	0	0.0
$1/(2\pi)$		0	0.0

*δ, Dirac delta function.
#Indicates where the probability is non-zero.

for some model probability functions $p(\Theta)$. The one-peaked $p(\Theta)$ have order parameters $S_D^2 > 0.68$, the two-peaked $p(\Theta)$ have $S_D^2 > 0.17$. Note that in Table 1 we used symmetric distributions; if one angle is populated more than another (e.g., 70% trans and 30% gauche$^+$), then the order parameters will have intermediate values.

As in the model-free approach of Lipari and Szabo, we approximate the correlation function $C(t)$ by a two-term exponential representation:

$$C(t) = S_D^2 + (1 - S_D^2)\exp(-t/\tau) \quad (7)$$

in which τ is a time constant, which is roughly equal to the residence time in a single dihedral angle conformation.

Our definition of a dihedral angle correlation function is different from that of Chandler (Chandler, 1978):

$$C(t) = \langle \delta N_T(t)\delta N_T(0)\rangle / \langle \delta N_T^2 \rangle \quad (8)$$

in which N_T is 1 for a trans configuration and 0 elsewhere, and $\delta N_T(t) = N_T(t) - \langle N_T \rangle$. In this approach a correlation time τ is defined as the integral from 0 to ∞ of the correlation function. A detailed analysis of the properties of this correlation function, and its application to computing dihedral transition rates from MD simulations, was presented by Zhang and Pastor (1994). Their study indicates that for short simulations the correlation times are dependent on the way in which the function is evaluated due to numerical problems, but they also present a formulation of Eq. 8 that resolves this problem.

The advantage of our definition (Eq. 1) is that it does not involve a definition of a trans configuration. Such a classification of torsion angles into discrete bins does not do justice to the nature of the dihedral probability distribution, which is a continuous function of the torsion angle. Furthermore, $C(t)$ from Eq. 1 is not sensitive to numerical problems like Eq. 8, and it may be averaged over many dihedrals for applications in lipids or polymers. Because our correlation function does not go to zero at infinite time, we have to use Eq. 7 to extract a correlation time.

METHODS

An L-Enk molecule (sequence Tyr-Gly-Gly-Phe-Leu) was built using QUANTA (MSI, 1994). The peptide was built in a bent conformation (Fig. 1) and in a linear conformation (Fig. 2) [plots were made using the MOLSCRIPT program (Kraulis, 1991)]. Both conformations were built in a zwitterionic form (with N-terminal NH_3^+ and C-terminal COO^- groups) and in neutral form (with N-terminal NH_2 and C-terminal COOH groups). The peptides were solvated in rectangular boxes with either water or DMSO molecules, by stacking equilibrated boxes of either solvent to form a box large enough to contain the peptide and 0.6 nm of solvent on all sides. All solvent molecules with any atom within 0.15 nm of the peptide were removed. After solvation, all configurations were energy minimized with the steepest descents method for 100 steps, to remove bad van der Waals contacts. With these peptides six simulations of 2.0 ns each were performed, using SPC water (Berendsen et al., 1981) or DMSO (Liu et al., 1995) as solvent. An overview of the simulations is given in Table 2. In DMSO both the zwitterionic and the neutral form of L-Enk were simulated, because they are both present in a ratio of 2:3 (Gerothanassis

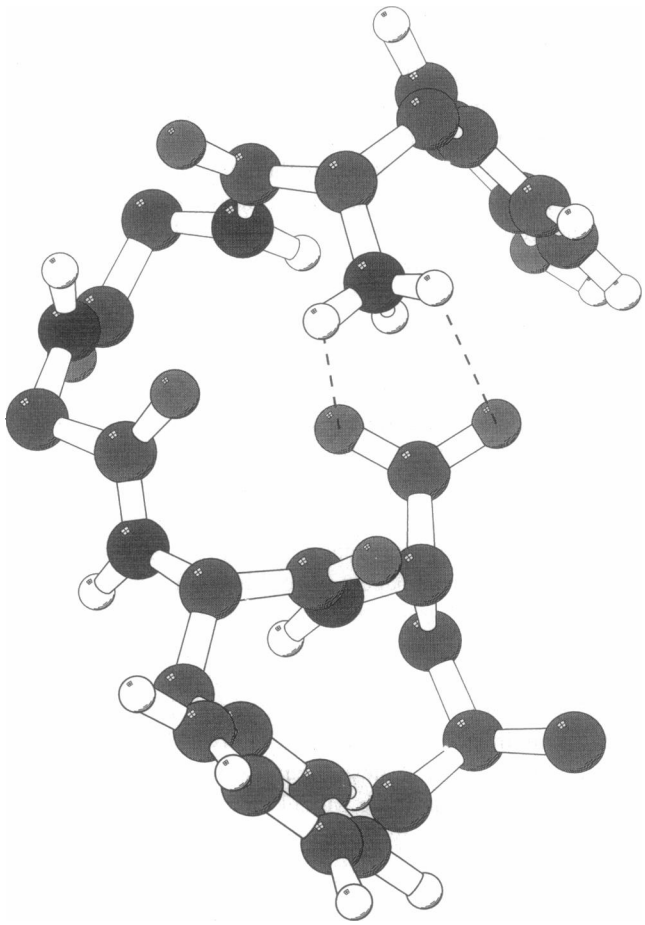

FIGURE 1 Starting structure of L-Enk in the bent conformation. The salt bridge is indicated by dashed lines.

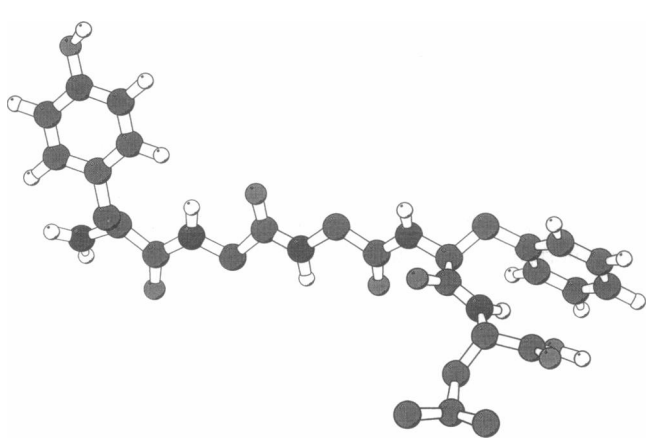

FIGURE 2 Starting structure of L-Enk in the linear conformation.

TABLE 2 Overview of the simulations performed, with starting conformation

Sim.	Description	Solvent	Conf.
Ebz+W	L-Enk	756 SPC	Bent
Elz+W	L-Enk	1177 SPC	Linear
Ebz+D	L-Enk (zwitterion)	210 DMSO	Linear
Ebn+D	L-Enk (neutral ends)	230 DMSO	Bent
Eln+D	L-Enk (neutral ends)	283 DMSO	Linear

et al., 1992). In water, only the zwitterionic L-Enk was simulated, because it is the normal form at pH 7 (Higashijima et al., 1979). For the peptide we used the GROMOS87 force field (van Gunsteren and Berendsen, 1987) with increased repulsion between water oxygen and carbon (van Buuren et al., 1993; Mark et al., 1994; Daura et al., 1996). The resulting parameter set is the one referred to as SW by Daura et al. (1996). Explicit hydrogen atoms were used for all polar groups plus the aromatic residues (van der Spoel et al., 1996b); united atoms were used for aliphatic hydrogens.

All simulations were performed with weak coupling (Berendsen et al., 1984) to a bath of constant temperature at 300 K, with coupling time τ_T of 0.1 ps. Peptide and solvent were coupled individually to the heat bath. Pressure coupling was also applied to a pressure bath with reference pressure of 1 bar, using a coupling time τ_P of 1.0 ps. A cutoff for nonbonded interactions of 1.0 nm was used in all simulations. The SHAKE algorithm (Ryckaert et al., 1977) was used to constrain all bond lengths, allowing an integration time step of 2 fs. For the water molecules we used the SETTLE algorithm to constrain the bond lengths as well as the bond angle (Miyamoto and Kollman, 1992). Neighbor lists were used and updated every 20 fs. The simulations were performed using the GROMACS software (van der Spoel et al., 1996a) on special-purpose parallel computers (Berendsen et al., 1995). The longest simulation (Elz+W) took 57 h on our 28-processor parallel computer.

RESULTS

Salt bridge between N- and C-terminus

We have calculated the distance between N- and C-terminal charged groups and the neutral equivalents in all simulations (Fig. 3). It can be seen that simulations Ebz+W and Ebn+D start out in a bent conformation in which the contact between N- and C-terminus is present. In Ebz+W the salt bridge seems to be water-mediated during the whole simulation, but the electrostatic interaction is maintained. The salt bridge is formed quickly in Elz+W after 200 ps, and remains rather stable during the remainder of the simulation. The zwitterionic form of L-Enk in DMSO starts out in a linear conformation, but after 750 ps the salt bridge is formed, and it remains intact for the remainder of the simulation. Both simulations of the neutral L-Enk in DMSO do not show any preferred interaction between N- and C-terminal groups.

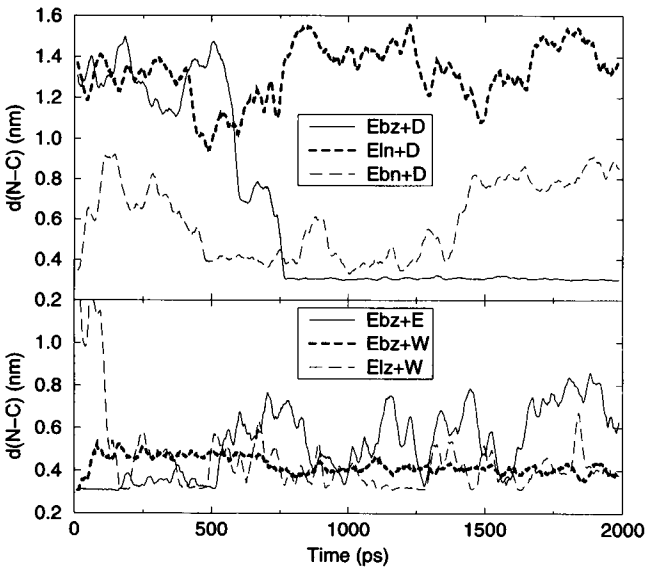

FIGURE 3 Distance between N- and C-terminal end groups (N resp. O atoms) in each simulation. A running average over 25 ps is given to improve clarity.

Secondary structure

The secondary structure of L-Enk in all simulations was determined using the DSSP program (Kabsch and Sander, 1983). In Fig. 4 the secondary structure is plotted as a function of time. Only bend- and turn-type structures were found during our simulations, which is not surprising because the peptide contains only five residues. The difference between a bend and a turn in the DSSP definition is that a turn should contain a hydrogen bond. It is clear that such hydrogen bonds are rare, although they do occur. In Table 3 we have printed the average proton donor-acceptor distances for the most important hydrogen bonds, including the salt bridge/hydrogen bond between Tyr^1 and Leu^5. In none of the simulations is there a permanent hydrogen bond involving Gly^2-CO or Gly^3-CO, but in sim. Elz+W both oxygens are hydrogen-bonded to Leu^5/NH for about half the simulation. There is also a hydrogen bond between Gly^2-NH and Leu^5-CO in Ebz+W and Ebz+D, but this hydrogen bond is absent in sim. Elz+W. Furthermore, it can be seen that the salt bridge is very strong in Ebz+D, whereas it is less so in aqueous solution.

Distance between aromatic rings

It was suggested by Gupta et al. (1986) that one of the Tyr-HD protons should be close to one of the Phe-HE protons, based on NOE measurements in water. From our water simulations we have calculated the shortest distance between the Tyr-HD and the Phe-HE protons at each time frame. We found the average distances to be 0.75 nm (Ebz+W) and 1.2 nm (Elz+W) which clearly are too large for an NOE.

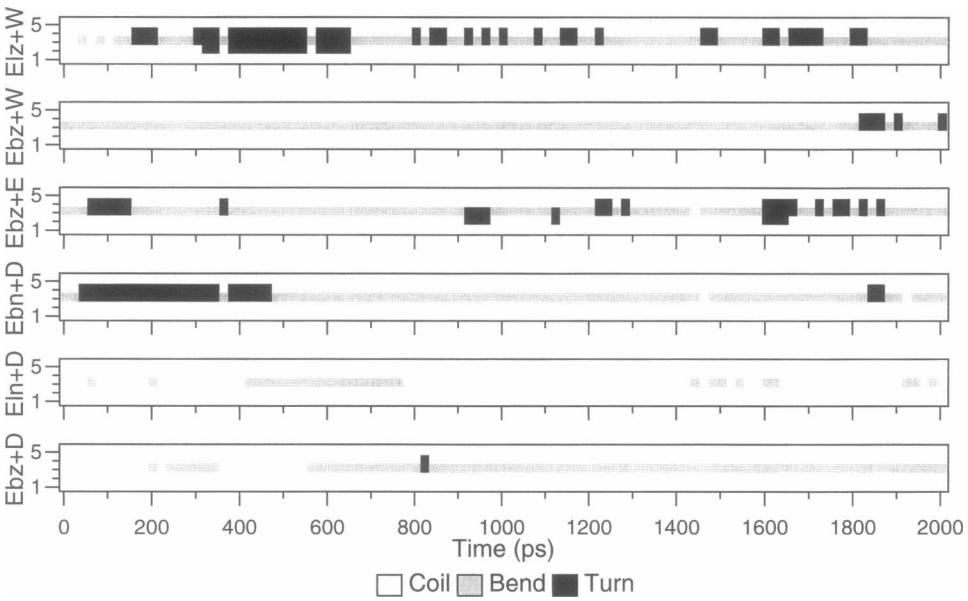

FIGURE 4 Secondary structure of L-Enk in our simulations.

Backbone conformation

To establish whether preferred conformations exist in solution we calculated Ramachandran plots for Gly^2, Gly^3, and Phe^4 (Fig. 5). Some appreciable differences are visible in the backbone angles. As expected, Phe^4 never has a positive Φ angle, while Gly^2 and Gly^3 do. Furthermore, the Phe^4 Ψ angle does not change in sim. Ebn+D, Eln+D, or Ebz+W. In Elz+W and Ebz+D both positive and negative Phe^4-Ψ are sampled, with a slight preference for negative values. In DMSO, the zwitterion in sim. Ebz+D samples two backbone conformations, first the linear one, with positive Ψ values for all residues, then the bent one, with negative values. The neutral peptides in DMSO are not very mobile; in both Ebn+D and Eln+D Phe^4 remains in its starting conformation, while Gly^3 has a preference for negative Ψ in Gly^3 in Ebn+D. The possible configurations for Gly^2 are sampled rather uniformly in these two simulations.

We also computed a correlation plot for the Gly^3-Φ and Gly^2-Ψ backbone angle in sim. Ebz+W (Fig. 6). A clear correlation between the two is visible, which means that these angles do not flip independently in sim. Ebz+W.

Side-chain conformations and interactions

The side chains in small peptides are very flexible. When there is no steric hindrance from neighboring residues, the rotation is limited only by the intra-residue interactions. The χ_1 dihedral angle can be in gauche$^-$ (+60°), trans (180°), or gauche$^+$ (−60°), with a preference for the latter value (Thornton, 1992). In contrast, amino acids in regular secondary structure elements, such as α-helix or β-sheet, rarely have side chains in the gauche$^-$ conformation (Thornton, 1992). In Fig. 7 we have plotted the distribution of χ_1 dihedral angles in DMSO and water, where we have averaged the distributions of the simulations in each solvent. In DMSO all side chains prefer the gauche$^+$ conformation, while in water the aromatic residues prefer the trans conformation, while Leu is mainly gauche$^+$. The other conformations do occur as well, and as expected, the gauche$^-$ is the least populated one in both solvents.

We have calculated the correlation times τ and dihedral order parameters S_D^2 from our simulations. In Fig. 8 the correlation functions $C(t)$ for all side-chain dihedral angles are plotted, averaged over the DMSO and water simulations. All correlation functions were calculated using Eq. 1, where we averaged over all starting points in the first 1000 ps, i.e., all points in the $C(t)$ were averaged over 2000 data points. It is clear from this figure that $C(t)$ for the Leu side chain have converged in both solvents, while it seems to be nearly converged for Phe in water as well. The other $C(t)$ have clearly not converged. In Table 4 we have printed the S_D^2 and τ values obtained by fitting Eq. 7 to the $C(t)$

TABLE 3 Average distance between donor and acceptor for the most important backbone hydrogen bonds

		⟨d(donor − acceptor)⟩ (nm)				
Donor	Acceptor	Ebz+W	Elz+W	Ebz+D	Eln+D	Ebn+D
Tyr^1-NH	Leu^5-CO	0.43	0.44	0.31	1.32	0.59
Gly^2-NH	Leu^5-CO	0.34	0.54	0.34	1.15	0.48
Gly^3-NH	Leu^5-CO	0.47	0.62	0.44	0.91	0.48
Leu^5-NH	Gly^2-CO	0.55	0.46	0.60	0.75	0.49
Leu^5-NH	Gly^3-CO	0.43	0.39	0.41	0.41	0.39

For Ebz+D averaging was done from 1000–2000 ps only, i.e., after formation of the salt bridge.

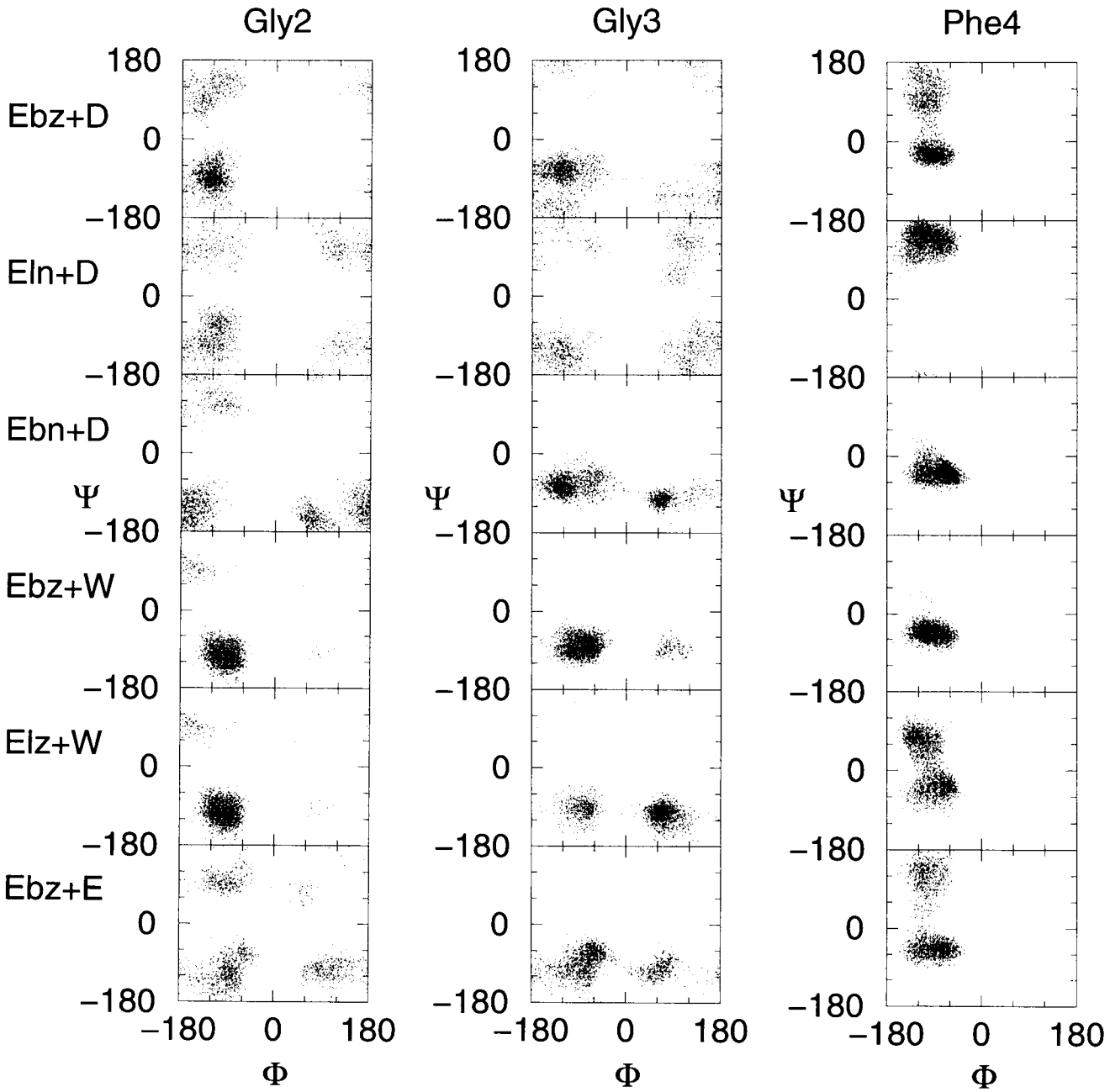

FIGURE 5 Ramachandran plots of Gly^2, Gly^3, and Phe^4 in all simulations.

functions, the S_D^2 calculated from the distribution using Eq. 5 and the number of dihedral transitions counted manually from the dihedral/time curve (data not shown). The order parameters calculated in both ways have converged for Leu (DMSO and water) and for Phe (water) while they do not in the other cases. This observation provides extra evidence that the latter side-chain motions have not equilibrated in our simulations.

DISCUSSION

The conformation of short linear peptides in solution remains a controversial topic. For some peptides transient conformations, predominantly β-turns, have been determined by NMR techniques (Dyson and Wright, 1991, 1993); such peptides have also been studied theoretically (Hermans, 1993). In another case, a strong interaction between a Tyr side chain and an amide proton was reported (Kemmink et al., 1993; van der Spoel et al., 1996b). In the case of the enkephalins, it was long thought that these peptides form a 2←5 β-turn as well. However, evidence from ^{17}O-NMR demonstrated that neither Gly^2-CO nor Gly^3-CO is hydrogen-bonded in water, DMSO, or acetone (Sakarellos et al., 1989; Moret et al., 1990). It can be expected that protons that are hydrogen-bonded permanently will have an upfield chemical shift. Despite the fact

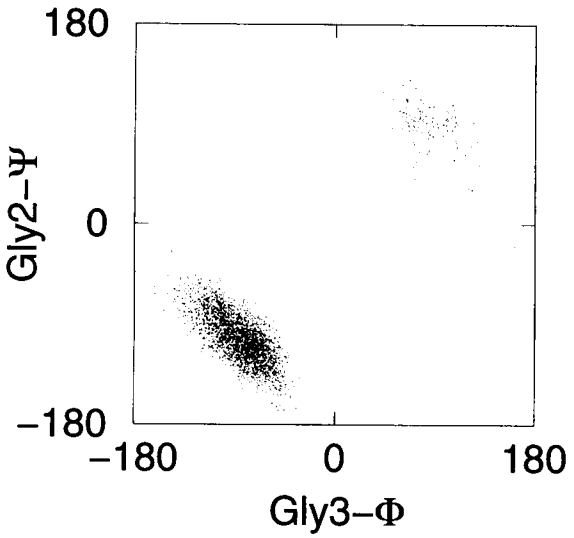

FIGURE 6 Correlation of Gly^3-Φ and Gly^2-Ψ angles in sim. Ebz+W

that there seems to be such a hydrogen bond from Gly^2-NH to Leu^5-CO in sim. Ebz+W and Ebz+D (Table 3), the experimental evidence does not support this unequivocally. Chemical shifts for Gly^2-NH were reported to be 8.59 in water and 8.78 in DMSO (Higashijima et al., 1979). For Gly^3-NH, however, there is an upfield shift of 0.5 ppm as compared to random coil values (8.00 in water versus 8.15 in DMSO). Such an upfield shift may also be induced by the aromatic rings, and therefore the experimental data cannot be regarded as hard evidence for hydrogen bonding of Gly^2-NH or Gly^3-NH (Higashijima et al., 1979).

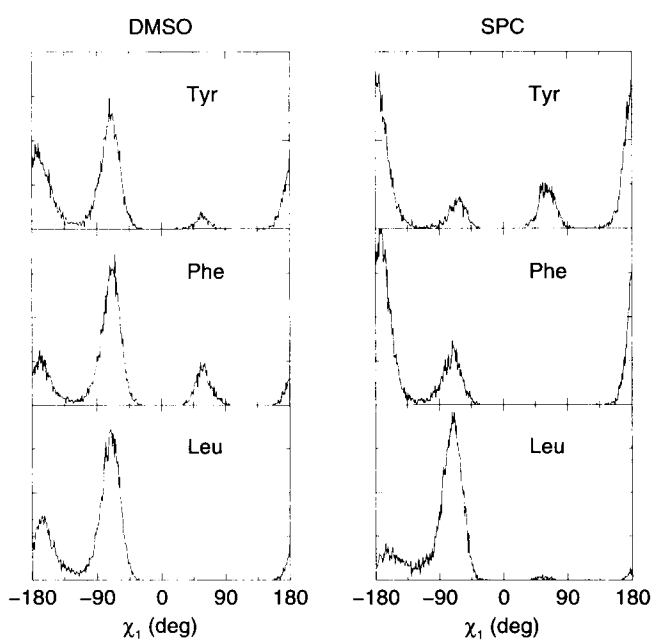

FIGURE 7 Distribution of χ_1 dihedral angles in DMSO (averaged over sims. Ebz+D, Eln+D, and Ebn+D) and SPC water (averaged over sims. Ebz+W and Elz+W).

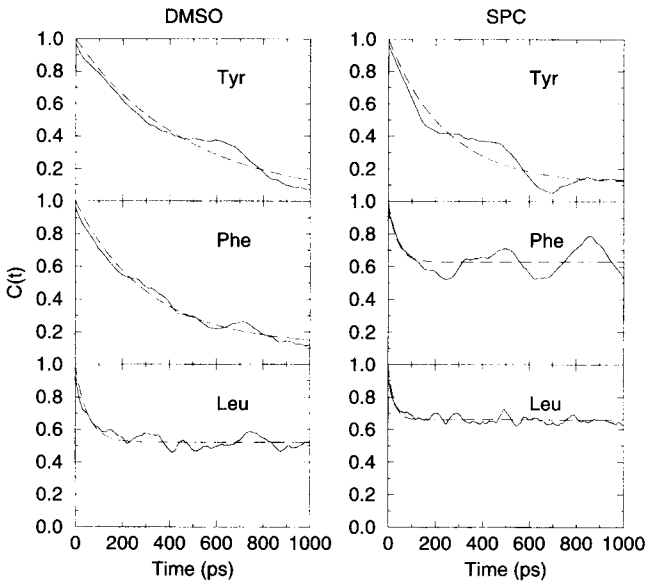

FIGURE 8 χ_1 Autocorrelation functions in DMSO (averaged over sims. Ebz+D, Eln+D, and Ebn+D) and SPC water (averaged over sims. Ebz+W and Elz+W). Solid line, simulation data; dashed line, fit to the data using Eq. 7.

Equilibration and peptide dynamics

In simulation studies it is important to look for evidence of equilibration. For simple systems such as pure liquids, this is usually not very hard, but for a single (macro)molecule in solution, it is. In general it is harder for peptides than for proteins, because peptides have considerable intrinsic flexibility. Some useful criteria in this context are potential energy and potential energy components, quantities that can be compared to experimental observables, correlation and distribution functions. The time-dependence of a secondary structure (Fig. 4) and of the distance between N- and C-termini of L-Enk (Fig. 3) are not very useful quantities when a single simulation is performed. We did, however, perform two simulations of L-Enk in water and two simulations of neutral L-Enk in DMSO with different starting conditions. From the Ramachandran plots (Fig. 5) it is clear that these simulations have not converged to the same Φ/Ψ distribution after 2.0 ns. The average distances for hydrogen bonds (Table 3) have not converged either.

In principle, the average over these simulations should yield a better representation of the equilibrium properties of L-Enk in DMSO or water. To test this, we have studied the side-chain dynamics of L-Enk in quite some detail and have calculated the χ_1 dihedral angle distributions (Fig. 7) and autocorrelation functions (Fig. 8). With the theory we have introduced in this paper, it is possible to calculate a property, the dihedral order parameter S_D^2, in two different ways. We find (Table 1) that for the Leu side chain the dihedral order parameters S_D^2 have converged in both DMSO and water, when calculated in the two different manners. In our opinion this strongly suggests that the Leu side-chain motions have equilibrated. Furthermore, we can deduce from Table 1 that dihedral transitions in (almost) random coil

TABLE 4

	SPC				DMSO			
Residue	S^2_D	τ(ps)	$S^2_D(p)$	trans. no.	S^2_D	τ(ps)	$S^2_D(p)$	trans. no.
TYR1	0.11	240	0.39	3	0.01	467	0.38	4
PHE4	0.63	37	0.62	7	0.11	306	0.39	10
LEU5	0.66	25	0.66	23	0.52	52	0.55	23

Order parameters S^2_D, time constants τ for χ_1 dihedral angles, order parameters based on the probability distribution $S^2_D(p)$ (see Fig. 7) and number of dihedral transitions, averaged over sims. Ebz+W and Elz+W (column SPC), respectively, sims. Ebz+D, Ebn+D, and Eln+D (column DMSO).

peptides take place on a time scale of 20 ps to 1 ns in both DMSO and water. The time scale of transitions seems to be strongly correlated to the size of the side chain. We have also computed the autocorrelation function of the Tyr^1-χ_1 angle for a period of 1200 ps in sim. Ebz+W where there is no dihedral transition. In this case the order parameter S^2_D is 0.93 and the correlation time is 4 ps. Thus, the correlation times that we have calculated are strongly dependent on the number of dihedral transitions, and therefore they are a good measure for the dihedral transition rate.

We conclude that neither the backbone dynamics of the L-Enk pentapeptide nor the side-chain motions for Tyr and Phe have fully equilibrated in 2.0 ns, which is in line with earlier simulation studies of short linear peptides (Tobias et al., 1991; Hermans, 1993; Brooks III and Case, 1993).

L-Enk in water

Although it has been shown that the choice of a water model may drastically influence the results from simulations (van der Spoel et al., 1996b) we decided to study L-Enk in SPC water (Berendsen et al., 1981). In general, the SPC/E model (Berendsen et al., 1987) is a better model for bulk water studies than SPC, but the SPC model was developed for studies of protein hydration, and it is widely used in protein and membrane simulations. It was found before that SPC/E used in interface studies produces interfaces that are too sharp (van Buuren et al., 1993), but in our previous work on a tetrapeptide from BPTI, we found that the SPC/E model was the only one to reproduce experimental chemical shift data (van der Spoel et al., 1996b). Thus, it is not possible to determine conclusively which one is the better model and we have to underline that very detailed analyses of simulation data are not warranted by the inaccuracies in the potential function. It is almost a cliche to state that water is the most important, yet the least understood molecule, but this explains why it is also the hardest molecule to model (Brodsky, 1996). For simulation work this means that details at water interfaces (including protein-water interfaces) are difficult to model, while the protein internal interactions, as well as bulk water properties, are probably described more accurately. Moreover, short peptides are even more difficult to model than proteins, as they are almost completely hydrated. Based on our experience, we prefer the SPC model for interface work, but we acknowledge that a significant improvement in the water model, such as the introduction of polarizability, is necessary in the near future.

Despite the clear hydrogen bond between Gly^2-NH and Leu^5-CO, the backbone of L-Enk seems to be flexible in Ebz+W, as shown by the Ramachandran plots (Fig. 5). However, when we look at Fig. 6 we see that the Gly^3-Φ and Gly^2-Ψ angles are correlated, which means that they always flip simultaneously. If we disregard the changes in Gly^2 and Gly^3 angles, we find that L-Enk is very stable in a single backbone conformation with all backbone angles in the α-helical part of Φ/Ψ space. Apparently the hydrogen bond is strong enough to keep Gly^2-NH and Leu^5-CO together. Since the latter groups are charged, this interaction is considerably stronger than a regular hydrogen bond. The starting conformation of L-Enk that we used for simulation Ebz+W was similar to the final conformation, which may have biased the result. Indeed, in the other SPC simulation (Elz+W) which started from a linear peptide conformation, there is no hydrogen bond between Gly^2-NH and Leu^5-CO. There is, however, a hydrogen bond between Gly^3-CO and Leu^5-NH, although it is present only 50% of the time, leading to an average distance of 0.39 nm. Although this seems to contradict results from ^{17}O-NMR, which showed that neither Gly^2-CO nor Gly^3-CO is hydrogen-bonded (Sakarellos et al., 1989; Moret et al., 1990), the hydrogen bond is not very strong, and therefore it will not have a profound influence on the chemical shift of the oxygen atoms. Moreover, both sim. Ebz+W and sim. Elz+W sample different conformations, and there will probably be substantial conformational averaging. Nevertheless, both conformations of L-Enk in water are compact, and can be characterized as a combination of bend- and turn-structures. It should be noted that where we find two different conformations in two simulations, another starting structure might generate yet another end conformation similar to the one found by Li and Scheraga using MCM (Li and Scheraga, 1987).

We could not find evidence for an interaction between aromatic groups in water, as suggested by Gupta et al. (1986). However, this was shown to be an artifact by Motta et al. (1987b), therefore it is not surprising that we did not find it.

L-Enk in DMSO

Neutral L-Enk in DMSO is very flexible; both Gly residues sample the complete Φ/Ψ space in Ebn+D as well as Eln+D, only Phe remains in its starting conformation, indicating that these two simulations have not converged in 2.0 ns. Apparently there is no driving force for neutral

L-Enk in DMSO to find a well-defined conformation. In contrast, the zwitterionic L-Enk in DMSO starts out in a linear conformation (Fig. 2), and after 750 ps it forms a salt bridge that is very stable (Fig. 3). These findings are in good agreement with experimental data that suggest that two different conformers are present in DMSO solution (Higashijima et al., 1979). Inasmuch as it was also determined that only 40% of the L-Enk molecules is actually a zwitterion (Gerothanassis et al., 1992), our results can account for the experimentally observation of two different conformations of L-Enk in DMSO solution; the zwitterionic in a bent form, the neutral in an extended form.

CONCLUSIONS

Somewhat to our surprise, we did find a preferential conformation for L-Enk from our simulation Ebz+W in SPC water. Therefore, we performed a second simulation (Elz+W) with another starting conformation. Both simulations find compact, rather stable conformations. The conformation in sim. Ebz+W is determined by a hydrogen bond from Gly^2-NH to the charged Leu^5-CO group, while in sim. Elz+W there is a rather strong hydrogen bond between Gly^3-CO and Leu^5-NH. It can not be ruled out however, that another starting conformation would yield yet another end conformation. In DMSO the final conformation of the zwitterionic L-Enk is very similar to the one in sim. Ebz+W, albeit that the salt bridge is much stronger, due to the lower dielectric constant of the solvent. The neutral L-Enk does not have a preferential structure, in accord with experimental data (Higashijima et al., 1979).

The χ_1 dihedral analysis (Fig. 7) shows that the amino acid side chains have considerable freedom, and sample the complete available χ_1 space (Thornton, 1992). Both the aromatic residues Tyr^1 and Phe^4 have a significant population of the gauche$^-$ conformation. In contrast, Leu^5 is almost never in the gauche$^-$ conformation, which may be due, in part, to the γ-branched side chain. Although Phe^4 and Tyr^1 have γ-branched side chains as well, the Leu side chain is different, because both Cδ atoms are in a tetrahedral conformation in contrast to the flat aromatic rings. There is no notable difference between side chain behavior in water and DMSO. The finding that all side chains individually sample the available χ_1 space renders a detailed description of low energy conformers in terms of dihedral angles meaningless (Perez et al., 1992). This last comment can also be applied to overly detailed determination of the structure of flexible regions in proteins, such as surface loops.

Finally, we would like to note that the dihedral analysis that we have introduced may provide useful information for the analysis of MD simulations of lipid molecules in membranes or micelles.

We would like to thank Dr. Hans Vogel for critical reading of the manuscript and Arjan van der Vaart for some initial analyses of the simulations.

This work was supported by the Netherlands Foundation for Chemical Research (SON) (to D.vdS.) with financial aid from the Netherlands Organization for Scientific Research (NWO).

REFERENCES

Berendsen, H. J. C., J. R. Grigera, and T. P. Straatsma. 1987. The missing term in effective pair potentials. *J. Phys. Chem.* 91:6269–6271.

Berendsen, H. J. C., J. P. M. Postma, A. DiNola, and J. R. Haak. 1984. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* 81:3684–3690.

Berendsen, H. J. C., J. P. M. Postma, W. F. van Gunsteren, and J. Hermans. 1981. Interaction models for water in relation to protein hydration. *In* Intermolecular Forces. B. Pullman, editor. D. Reidel Publishing Company, Dordrecht, The Netherlands. 331–342.

Berendsen, H. J. C., D. van der Spoel, and R. van Drunen. 1995. GROMACS: A message-passing parallel molecular dynamics implementation. *Comp. Phys. Comm.* 91:43–56.

Bodkin, M. J., and J. M. Goodfellow. 1996. Hydrophobic solvation in aqueous trifluoroethanol solution. *Biopolymers.* 39:43–50.

Brodsky, A. 1996. Is there predictive value in water computer simulations? *Chem. Phys. Lett.* 261:563–568.

Brooks III, C. L., and D. A. Case. 1993. Simulations of peptide conformational dynamics and thermodynamics. *Chem. Rev.* 93:2487–2502.

Brooks III, C. L., and L. Nilsson. 1993. Promotion of helix formation in peptides dissolved in alcohol and water-alcohol mixtures. *J. Am. Chem. Soc.* 115:11034–11035.

Chandler, D. 1978. Statistical mechanics of isomerization dynamics in liquids and the transition state approximation. *J. Chem. Phys.* 68: 2959–2970.

Daura, X., B. Oliva, E. Querol, F. X. Aviles, and O. Tapia. 1996. On the sensitivity of MD trajectories to changes in water-protein interaction parameters: the potato carboxypeptidase inhibitor in water as a test case for the GROMOS force field. *Proteins Struct. Funct. Genet.* 25:89–103.

de Loof, H., L. Nilsson, and R. Rigler. 1992. Molecular dynamics simulations of galanin in aqueous and nonaqueous solution. *J. Am. Chem. Soc.* 114:4028–4035.

Doi, M., A. Ishibe, H. Shinozaki, H. Urata, M. Inoue, and T. Ishida. 1994. Conserved and novel structural characteristics of enantiomorphic Leu-enkephalin. *Int. J. Pept. Prot. Res.* 43:325–331.

Dyson, H. J., and P. E. Wright. 1991. Defining solution conformations of small linear peptides. *Annu. Rev. Biophys. Biophys. Chem.* 20:519–538.

Dyson, H. J., and P. E. Wright. 1993. Peptide conformation and protein folding. *Curr. Opin. Struct. Biol.* 3:60–65.

Garbay-Jaureguiberry, C., B. P. Roques, R. Oberlin, M. Anteunis, S. Combrisson, and J. Y. Lallemand. 1977. ^{1}H and ^{13}C-NMR studies of conformational behaviour of Leu-enkephalin. *FEBS Lett.* 76:93–98.

Gerothanassis, I. P., N. Birlirakis, T. Karayannis, V. Tsikaris, M. Sakarellos-Daitsiotis, C. Sakarellos, B. Vitous, and M. Marraud. 1992. ^{17}O-NMR and FTIR study of the ionization state of peptides in aprotic solvents. *FEBS Lett.* 298:188–190.

Gerothanassis, I. P., T. Karayannis, M. Sakarellis-Daitsiotis, C. Sakarellos, and M. Marraud. 1987. Nitrogen-14 nuclear magnetic resonance of the amino terminal group of Leu-enkephalin in aqueous solution. *J. Magn. Reson.* 75:513–516.

Glasser, L., and H. A. Scheraga. 1988. Calculations on crystal packing of a flexible molecule, Leu-enkephalin. *J. Mol. Biol.* 199:513–524.

Graham, W. H., E. S. Carter II, and R. P. Hicks. 1992. Conformational analysis of Met-enkephalin in both aqueous solution and in the presence of sodium dodecyl sulfate micelles using multidimensional NMR and molecular modelling. *Biopolymers* 32:1755–1764.

Gupta, G., M. H. Sarma, R. H. Sarma, and M. M. Dhingra. 1986. NOE data at 500 MHz reveal the proximity of phenyl and tyrosine rings in enkephalin. *FEBS Lett.* 198:245–250.

Han, S. L., E. R. Stimson, F. R. Maxfield, S. J. Leach, and H. A. Scheraga. 1980. Study of the state of ionization of [Leu^5]-enkephalin in the crystal and in solution. *Int. J. Pept. Prot. Res.* 16:183–190.

Hermans, J. 1993. Molecular dynamics simulations of helix and turn propensities in model peptides. *Curr. Opin. Struct. Biol.* 3:270–276.

Higashijima, T., J. Kobayashi, and T. Miyazawa. 1979. Nuclear magnetic resonance study on Met-enkephalin and Met-enkephalinamide. *Eur. J. Biochem.* 97:43–57.

Hughes, J., T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, and H. R. Morris. 1975. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature.* 258:577–579.

Isogai, Y., G. Nemethy, and H. A. Scheraga. 1977. Enkephalin: conformational analysis by means of empirical energy calculations. *Proc. Natl. Acad. Sci. U.S.A.* 74:414–418.

Kabsch, W., and C. Sander. 1983. Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers.* 22:2577–2637.

Karoslaw, J., and H. A. Scheraga. 1992. Application of the diffusion equation method for global optimization to oligopeptides. *J. Phys. Chem.* 96:7442–7449.

Kemmink, J., C. P. M. van Mierlo, R. M. Scheek, and T. E. Creighton. 1993. Local structure due to an aromatic-amide interaction observed by ^{1}H-nuclear magnetic resonance spectroscopy in peptides related to the N-terminus of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* 230: 312–322.

Khaled, M. A., M. M. Long, W. D. Thompson, R. J. Bradley, G. B. Brown, and D. W. Urry. 1977. Conformational states of enkephalins in solution. *Biochem. Biophys. Res. Commun.* 76:224–231.

Kraulis, P. J. 1991. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Cryst.* 24:946–950.

Li, Z., and H. A. Scheraga. 1987. Monte carlo-minimization approach to the multiple minima problem in protein folding. *Proc. Natl. Acad. Sci. U.S.A.* 84:6611–6615.

Lipari, G., and A. Szabo. 1982. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 1. Theory and range of validity. *J. Am. Chem. Soc.* 104:4546–4559.

Liu, H., F. Muller-Plathe, and W. F. van Gunsteren. 1995. A force field for liquid dimethyl sulfoxide and liquid properties of liquid dimethyl sulfoxide calculated using molecular dynamics simulation. *J. Am. Chem. Soc.* 117:4363–4366.

Mark, A. E., S. P. van Helden, P. E. Smith, L. H. M. Janssen, and W. F. van Gunsteren. 1994. Convergence properties of free energy calculations: α-cyclodextrin complexes as a case study. *J. Am. Chem. Soc.* 116:6293–6302.

Meirovitch, H., E. Meirovitch, A. Michel, and M. Vaśquez. 1994. A simple and effective procedure for conformational search of macromolecules: application to Met- and Leu-enkephalin. *J. Phys. Chem.* 98:6241–6243.

Mierke, D. F., and H. Kessler. 1991. Molecular dynamics with dimethyl sulfoxide as a solvent. Conformation of a cyclic hexapeptide. *J. Am. Chem. Soc.* 113:9446.

Mierke, D. F., and H. Kessler. 1993. Improved molecular dynamics simulations for the determination of peptide structures. *Biopolymers.* 33: 1003–1017.

Miyamoto, S., and P. A. Kollman. 1992. SETTLE: an analytical version of the SHAKE and RATTLE algorithms for rigid water models. *J. Comp. Chem.* 13:952–962.

Moret, E., I. P. Gerothanassis, R. N. Hunston, and J. Lauterwein. 1990. Does a 2 ← 5 β-turn structure exist in enkephalins? *FEBS Lett.* 262: 173–175.

Motta, A., D. Picone, T. Tancredi, and P. A. Temussi. 1987. NOE measurements on linear peptides in cryoprotective aqueous mixtures. *J. Magn. Reson.* 75:364–370.

Motta, A., T. Tancredi, and P. A. Temussi. 1987a. Nuclear overhauser effects in linear peptides. *FEBS Lett.* 215:215–218.

Motta, A., D. Picone, T. Tancredi, and P. A. Temussi. 1988b. Low temperature NMR studies of Leu-enkephalins in cryoprotective solvents. *Tetrahedron.* 44:975–990.

MSI 1994. QUANTA 3.0. Molecular Simulations Incorporated, York, United Kingdom.

Nayeem, A., J. Villa, and H. A. Scheraga. 1991. A comparative study of the simulated-annealing and Monte Carlo-with-minimization approaches to the minimum-energy structures of polypeptides: [Met]-enkephalin. *J. Comp. Chem.* 12:594–605.

Paine, G. H., and H. A. Scheraga. 1985. Prediction of the native conformation of a polypeptide by a statistical-mechanical procedure. I. Backbone structure of enkephalin. *Biopolymers.* 24:1391–1346.

Paine, G. H., and H. A. Scheraga. 1986. Prediction of the native conformation of a polypeptide by a statistical-mechanical procedure. II. Average backbone structure of enkephalin. *Biopolymers.* 25:1547–1563.

Paine, G. H., and H. A. Scheraga. 1987. Prediction of the native conformation of a polypeptide by a statistical-mechanical procedure. III. Probable and average conformations of enkephalin. *Biopolymers.* 26: 1125–1162.

Perez, J. J., G. H. Loew, and H. O. Villar. 1992. Conformational study of met-enkephalin in its zwitterionic form. *Int. J. Quant. Chem.* 44: 263–275.

Picone, D., A. D'Ursi, A. Motta, T. Tancredi, and P. A. Temussi. 1990. Conformational preferences of [Leu^5]enkephalin in biomimetic media. *Eur. J. Biochem.* 192:433–439.

Ryckaert, J. P., G. Ciccotti, and H. J. C. Berendsen. 1977. Numerical integration of the Cartesian equations of motion of a system with constraints; molecular dynamics of n-alkanes. *J. Comp. Phys.* 23: 327–341.

Sakarellos, C., I. P. Gerothanassis, N. Birlirakis, T. Karayannis, and M. Sakarellos-Daitsiotis. 1989. ^{17}O-NMR studies of the conformational and dynamics properties of enkephalins in aqueous and organic solutions using selective labeled analogues. *Biopolymers.* 28:15–26.

Smith, P. E., and B. M. Pettitt. 1991a. Peptides in ionic solutions: a comparison of the ewald and switching function techniques. *J. Chem. Phys.* 95:8430–8441.

Smith, P. E., and B. M. Pettitt. 1991b. Effects of salt on the structure and dynamics of the bis(penicillamine) enkephalin zwitterion: a simulation study. *J. Am. Chem. Soc.* 113:6029–6037.

Stimson, E. R., Y. C. Meinwald, and H. A. Scheraga. 1979. Solution conformation of enkephalin. A nuclear magnetic resonance study of ^{13}C-enriched carbonyl carbons in [Leu^5]-enkephalin. *Biochemistry.* 18: 1661–1671.

Surewicz, W. K., and H. H. Mantsch. 1988. Solution and membrane structure of enkephalins as studied by infrared spectroscopy. *Biochem. Biophys. Res. Commun.* 150:245–251.

Thornton, J. M. 1992. Protein structures: the endpoint of the folding pathway. *In* Protein Folding. T. E. Creighton, editor. Freeman, New York. 59–81.

Tobias, D. J., J. E. Mertz, and C. L. Brooks III. 1991. Nanosecond timescale folding dynamics of a pentapeptide in water. *Biochemistry.* 30:6054–6058.

van Buuren, A. R., and H. J. C. Berendsen. 1993. Molecular dynamics simulation of the stability of a 22 residue alpha-helix in water and 30% trifluoroethanol. *Biopolymers.* 33:1159–1166.

van Buuren, A. R., S. J. Marrink, and H. J. C. Berendsen. 1993. A molecular dynamics study of the decane/water interface. *J. Phys. Chem.* 97:9206–9212.

van der Spoel, D., A. R. van Buuren, E. Apol, P. J. Meulenhoff, D. P. Tieleman, A. L. T. M. Sijbers, R. van Drunen, and H. J. C. Berendsen. 1996a. Gromacs User Manual version 1.3. Nijenborgh 4, 9747 AG Groningen, The Netherlands. Internet: http://rugmd0.chem.rug.nl/~gmx.

van der Spoel, D., A. R. van Buuren, D. P. Tieleman, and H. J. C. Berendsen. 1996b. Molecular dynamics simulations of peptides from BPTI: a closer look at amide-aromatic interactions. *J. Biomol. NMR.* 8:229–238.

van Gunsteren, W. F., and H. J. C. Berendsen. 1987. Gromos-87 manual. Biomos BV Nijenborgh 4, 9747 AG Groningen, The Netherlands.

Vesterman, B., J. Saulitis, J. Betins, E. Liepins, and G. V. Nikiforovich. 1989. Dynamic space structure of the Leu-enkephalin molecule in DMSO solution. *Biochim. Biophys. Acta.* 998:204–209.

Zhang, Y., and R. W. Pastor. 1994. A comparison of methods for computing transition rates from molecular dynamics simulation. *Mol. Sim.* 13:25–38.

Zhorov, B. S., and V. S. Ananthanarayanan. 1994. Similarity of Ca^{2+}-bound conformations of morphine and Met-enkephalin: a computational study. *FEBS Lett.* 354:131–134.